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BIOTECHNOLOGICAL PRODUCTION OF RECOMBINANT TISSUE PLASMINOGEN ACTIVATOR PROTEIN & ITS APPLICATION

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ABSTRACT

Biopharmaceuticals based on proteins, antibodies or nucleic acids are progressively used for the treatment of various diseases. This review discusses Tissue plasminogen activator (tPA or PLAT) as a biopharmaceuticals product, its synthesis, and administration of disease. Tissue plasminogen activator is a serine protease. It plays the role of conversion of plasminogen to plasmin. It is the major enzyme, responsible for the breakdown of the clot. In order to fulfill the acute demand of this product in the market, one of the premier methods is the biotechnological production of recombinant tissue plasminogen activator protein (reteplase) by transplastomic tobacco cell cultures. Other methods of production include,* Human *Tissue Plasminogen Activator (t-PA) c-DNA active expression from Pulmonary Metastases in the Methylotrophic Yeast Pichia PastorisKM71H, Strain and expression of Human Tissue Plasminogen, Activator in Escherichia coli using cytoplasmic and periplasmic cumulative Power. These methods describe the various aspects related to tPA, and have potential ability to produce tissue plasminogen activator protein.

KEYWORDS*: *Tissue Plasminogen Activator Protein (Reteplase), Transplastomic Tobacco Cell Cultures, Cytoplasm, Periplasm & Tissue Plasminogen Activator- Pichia Pastoris

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Original Article

INTRODUCTION

Tissue plasminogen activator is a type of protein that dissolves blood clots. It can be considered as a medication and known as a thrombus. Biochemically, tissue plasminogen activator is a serine protease which catalyzes the conversion of plasminogen to plasmin. It is found in endothelial cells of blood vessels and used as clinical medicine to treat thrombotic stroke. It is an intravenous or IV medication, usually given through a catheter inserted into a vein in the arm. They have been used in contraindicated head trauma and hemorrhagic stroke.

Recombinant biotechnology techniques can be used to manufacture Tissue plasminogen activator. Alteplase, Tenecteplase, Reteplase are specific recombinant tissue plasminogen activator (rtPA). The main biotechnological systems to produce recombinant biopharmaceuticals is based on microorganism cultures such as *Escherichia coli* and yeast at bioreactor level, while large proteins generally produced by mammalian cell platforms (31). About 60 peptides have been approved by the US FDA to date, more than 140 are under clinical study and by 2020, the global sales of biopharmaceuticals are expected to be worth over $US 278.2 billion (32).

Medical uses of Tissue Plasminogen Activator Protein

Pulmonary embolism, stroke, myocardial infarction are diseases that feature blood clots and tPA is used in these cases, in a medical treatment called thrombolysis. tPA was approved by the FDA in 1996 and commonly used in treatment of ischemic type strokes. In the case of an acute myocardial infarction, acute ischemic stroke, it is administered, and some administration is through an arterial catheter, directly to the site of occlusion in the peripheral arterial thrombi and thrombi in the proximal deep veins of the leg (5).

In case of the acute ischemic stroke, twelve large-scale and high-quality trials of rtPA have been carried out. Transplastomic technology has been used to produce recombinant proteins, and it enhanced field-grown plant resistance to herbicides and plagues. However, the derived cell cultures have been scarcely applied for the biotechnological production of heterologous proteins (33). Transplastomic plants have been targeted to produce biopharmaceuticals due to the high number (approx. 100) of chloroplasts per plant cell, the high copy number (approx. 10,000) of the plastid genome, as well as the maternal mode of inheritance. However, low-level leakages of transgenes in pollen may occur (34). The biotechnological production of recombinant tissue plasminogen activator protein (reteplase) by transplastomic tobacco cell cultures was successfully carried out (1). Reteplase is highly fibrin-specific (35).

In Protocol guidelines, it is used intravenously within the first three hours of the event. The Canadian Stroke Network guidelines recommend that, patients with ischemic stroke should be given treatment within four and a half hours of appearance of symptoms. They should also be evaluated for their eligibility to undergo treatment with Tpa (8). The current guidelines indicate that treatment of ischemic stroke can be carried out within four and a half hours, instead of earlier recommended three hours. But, this treatment depends on patient's medical history and present medical condition and complications (10).

It has greater thrombolytic potency of 4 folds and prolonged half-life of 18 min than alteplase. The longer half-life of the medicine permits its administration as a double bolus, if given 30 min apart(36-40). The necessity of a continuous intravenous infusion avoids by this arrangement (37).

General Consensus on Use

Generally, there is a debate in the emergency medicine community, regarding recombinant tPA's effectiveness in ischemic stroke. Most of the stroke specialists agree that tPA is the best medication for stroke patients. Also, it has been observed that benefits of tPA therapy are far more than the risks associated with it. In case of pulmonary embolism, a condition that arises when the blood clots move to the pulmonary arteries, the medication recommended by most of the specialists is the recombinant tPA (19-21). In a study, most of the patients treated with tPA as a medication for acute ischemic stroke recovered and survived. The people suffering from acute frostbite when treated with tPA, survived better with few amputations, while those patients who were not treated with tPA showed poor survival rate (13-15).

The first tPA was produced by recombinant DNA techniques at Genentech in 1982.Tissue-type plasminogen activators were identified and isolated from mammalian tissues and established a cDNA Library by use of reverse transcriptase and mRNA isolate from human melanoma cells. Screening of the cDNA library as well as sequence analysis was carried out. The sequences were compared to a whole genome library to confirm the presence of a specific protein. Later, the protein would be isolated with high accuracy and purity.

A synthetic plasmid was used to clone the cDNA sequence of interest. The sequence was expressed in E. coli cells and later in yeast cells. The expressed protein was checked for purity, stability etc. and finally expression was carried out in mammalian cells for mass scale production of the recombinant protein. Industrial scale fermenters were used for this purpose.

Some commercial recombinant tissue plasminogen activators are described in the form of table1:

Table 1: Description and Administration of few Recombinant Tissue Plasminogen Activator Proteins

Name of products	Drug Description
1) Activase (Alteplase)	It is produced by recombinant DNA technology. It is synthesizing by using the complementary DNA (cDNA), natural human tissue-type plasminogen activator obtained from a human melanoma cell line. Activase is a sterile and purified glycoprotein of 527 amino acids, lyophilized powder for intravenous administration.
2) Tenecteplase	Tenecteplase is used to prevent death in people who have had a heart attack (acute myocardial infarction), and it is also a recombinant tissue plasminogen activator or glycoprotein of 527 amino acids.
3) Reteplase	It is a non-glycosylated deletion mutein of tPA containing the kringle2. It is approved by FDA for acute myocardial infarction, because it is more convenient administration than alteplase and faster thrombolysis also. Its half-life is up to 20 minutes which allows it to be administered as a bolus injection.

BIOTECHNOLOGICAL PRODUCTION OF RECOMBINANT TISSUE PLASMINOGEN ACTIVATOR PROTEIN (RETEPLASE) FROM TRANSPLASTOMIC TOBACCO CELL CULTURES

One of the impressive methods of biotechnological production of recombinant tissue plasminogen activator protein (reteplase) is the transplastomic tobacco cell culture. Transplastomic plants are helpful for the mass production of biopharmaceuticals, because of many reasons. The most important are polyploidy of the plastid genome, low risk of pollen-mediated out crossing and maternal inheritance. Recently, by making use of genetic engineering techniques, K2S gene coding for human tPA protein having high biological activity was introduced into tobacco plants. Successful introgression of the gene in the transplantonic tobacco lines produced recombinant protein in the leaves of the plant at optimum levels. This was a great boost to the biopharmaceutical industry, as it led to the production of recombinant plasminogen activator protein in plant cell culture-based fermenters. The production of the protein was also standardized by providing light and dark conditions to cell cultures, and nearly three times increase in the expression of protein was observed in the light condition, as compared to dark (1)

Five different notable domains of tPA are N terminal finger, epidermal growth factor, a serine protease, Kringle 1 and Kringle 2 (41). The active part of the protein is thrombolytic KRingle 2 domain, which is a serine protease. The two functional regions of the protease are 176-527 aa residues and 1-3 aa in the n-terminal of the protein. The latter is often known as the truncated human tissue plasminogen activator or K2S, reteplase. It has longer plasma

half-life and exhibits higher fibrinolytic activity than tPA (42). Molecular farming can be utilized for biopharmaceutical production because transgenic plants need water, sunlight, minerals for growth. According to the US Agricultural Department, major drawback of the system are a lack of guaranteed trans-gene contention and the risk of contamination of the human food chain, if edible plant species are used as the host (43). Despite all the restraints, tissue-type plasminogen activator (tPA), however is an important target for biotechnological production by molecular farming.

The production and purification of truncated human tissue plasminogen activator protein produced from tobacco transplastomic plants (45) have been optimized, achieving a production of up to 30.6 mg/100 mg fresh weight of leaf tissue (46). In the study, transgenic cell suspension cultures and K2S gene expression system establishment took place after 2 months of the initial callus induction. Sufficient material was obtained to establish the cell suspension culture. Study parameters included growth index and biomass productivity (rx). Growth index (GI) is a measure of growth capacity. It means, harvested fresh weight/inoculum fresh weight. Lightdid not affect the biomass production of the system, and in both conditions i.e., light or darkness, the cell cultures reached a GI > 3, which represents a rx> 18.5 g L^{-1} d^{-1}. Tobacco suspension cultures showed the high capacity of the system to produce biomass, which was not affected even by the K2S gene expression.

cp value of the housekeeping genes (EF1 and accD) were analyzed by qPCR both in combination as well as separately to calculate and compare the CV between them, and to normalize the gene expression data more precisely. The results, CVEF1 ¼ 0.0247 < CVEF1&accD ¼ 0.0307 <CVaccD ¼ 0.0513, signify that the accD gene expression was affected in presence of light.

Mass spectroscopy was also done for the rtPA heterologous protein characterization. In all samples, the most intensive signals were detected for the peptides GGLFADIASHPWQAAIFAK and VYTAQNPSAQALGLGK, at a retention time of 3.4 min and 2.0 min, respectively. The results thus obtained affirmed that the K2S transgenic cell line is a promising line, and can be used to produce the recombinant protein in industrial scale.

Determination of the Protein Content and Activity Assay of Protein

TSP before purification can be used to measured estimate recombinant protein, which is expressed in the chloroplasts of the cell aspolyhistidine-taggedreteplase (45). Recombinant tissue plasminogen activator protein content in the tobacco cell suspension was 0.083% of the TSP when cultivated in darkness, rising to 0.277% in light conditions. That's why, result represents an increase of more than 3-fold (1). Therefore, the protein was purified from the cell suspension culture after a period of 8 weeks, instead of period of 2 weeks. Previous reports show that the production of recombinant protein in plant cell suspensions is lower than in the whole plant (47, 48, 49, 50).

In plastid genome, astrong sigma70-type rRNA operon (Prrn) promoter is recognized by the plastid-encoded RNA polymerase (51). It is reported that Prrn, a constitutive promoter in chloroplasts and prokaryotes can cause high levels of transcript accumulation in the plastids (47). Some factors are responsible for lower reteplase production in cell cultures. These factors may be less differentiated cells and plastids (53). The recombinant protein secreted to the culture medium may be un-stable. The instability of the protein may be due to the effect of proteases, which are also secreted from disrupted cells (48). Lower production of the protein may be the outcome of aggregation. The problem of aggregation arises, when the daughter cells fail to separate after cell division in the culture medium. (32)

Nevertheless, the rt-PA yield was significantly higher than the tPA production (up to 0.017%) in a hairy root system (54). Unlike tPA, reteplase has no carbohydrate side chains, and thus can be produced in E. coli cells, but most of the protein is present as an inclusion body, making its extraction and renaturation a time to consume downstream process, involving solubilization and protein refolding and dialysis (55). The culture medium could be optimized e.g. by increasing aeration (56) or adding growth medium supplements such as amino acids (57), gibberellic acid, haemin (58), a carbon source (32), protease substrates like gelatin, biopolymers (59, 60) or bovine serum albumin (61, 62).

For the quantitative measurement of reteplase activity, the extracted native protein was obtained from a cell suspension growing in light, since the production was higher than in darkness (1). A suitable and better approach to enhance the productivity of the K2S tobacco cell suspensions by modifying the promoter and vector regulatory elements (48). It minimizes the processing time and avoids proteolytic and oxidative degradation of recombinant protein (57). Addition of signal peptides like 33KDsp may improve secretion efficiency (63). Fungal hydrophobin as a fusion tag (64) and fusion tag like zein-derived peptides could increase the accumulation of proteins (65).

The results indicate that, the extraction of reteplase in native conditions preserves its biological activity. Thus, this biotechnological system could be used successfully to produce the biopharmaceutical.

HUMAN TISSUE PLASMINOGEN ACTIVATOR (T-PA) C-DNA ACTIVE EXPRESSION FROM PULMONARY METASTASES IN THE METHYLOTROPHIC YEAST PICHIA PASTORISKM71H STRAIN

Functional full-length human t-PA was expressed in KM71H host strain. Human protein t-PA has a complex structure, and its production in prokaryotic hosts have encountered various challenges including low production yield, inclusion body formation, misfolding and lack of the activity (66, 67).

Hyper glycosylation, improper folding and poor export, hamper the production of active t-PA in *Saccharomyces cerevisiae* and insect cells (68, 69). Recently, methylotrophic yeasts, particularly *Pichia pastoris* in the biopharmaceutical industry have been found as one of the best candidates for production of mammalian recombinant proteins, because of their ability to achieve many eukaryotic posttranslational modifications (70, 71, 72, and 73).

GS115 and KM71H are two strains of *P.pastoris*, which are available for recombinant protein production (74, 75). Since AOX1 gene has been disrupted in KM71H, the growth of this strain in the presence of methanol is slower than GS115 (76, 77). The unique *Pichia pastoris* expression system to produce recombinant proteins produces a high level of recombinant protein. In a study of evaluation of the t-PA expression in KM71H strains, where the cDNA of the t-PA gene was amplified by PCR, sequenced and cloned into *Pichia pastoris* KM71H host strain using pPICZ-alpha A expression vector. The expression system allowed methanol-induced expression and secretion of the protein (8). The PCR product was cloned in T-vector. The sequence was sequenced and analyzed using CLC sequence viewer software (version 6). There was no mutation in the cloned gene and the gene was found to be in its correct orientation in the expression vector for production of the recombinant protein (79).

The creation of the recombinant *Pichia pastoris* KM71H by integration of the expression cassette into the host strain, which was resistant to zeocin and could grow on YPDS medium supplemented with a zeocin antibiotic. PCR analysis of genomic DNA of the recombinant colony revealed the existence of the t-PA.

The confirmed recombinant colonies were subjected for recombinant protein production in BMMY medium. Growth curve of cells, transformed by pPICZ-alpha A-t-PA under induction of 0.5% methanol, showed a progressive increase in growth of the recombinant *Pichia pastoris* in the expression medium.

Maximum growth was obtained at 144 hours after inoculation. Dot blotting test revealed the expression of the recombinant t-PA in the culture supernatants. Level of the recombinant protein production at different time points was not equal while the highest level of the protein expression happened at 144 h (79).

Using quantitative ELISA, A450 of the standard Human t-PA dilution series and the readings of test samples were calculated. According to concentrations of the standard Human t-PA dilution series against of A450, a standard curve was depicted with $R^2 = 0.9985$. Based on the standard curve, the quantity amounts of the extracellular recombinant t-PA was determined to be 810.79 μg/L (8).

The approximate size of 70 kDa for recombinant t-PA was revealed by Western blotting test. The proteolytic activity and biological function of the expressed recombinant t-PA were confirmed by Zymography analysis. The expression medium supernatants had been applied in native condition (without boiling), showed white band in the dark blue background due to the production of plasmin with cleavage of plasminogen by the recombinant t-PA. The boiled supernatant sample did not generate white band in the presence of plasmin. The results confirmed biological and proteolytic activity of the recombinant t-PA.

Thus, *Pichia pastoris* KM71H proved to be an appropriate strain for production of active recombinant protein.

EXPRESSION OF HUMAN TISSUE PLASMINOGEN ACTIVATOR IN *ESCHERICHIA COLI*, USING CYTOPLASMIC AND PERIPLASMIC CUMULATIVE POWER

The expression of complex proteins with multiple disulfide bridges is one of the most important limitations for high yield heterologous protein production in *Escherichia coli*. Using a PelB signal peptide sequence at 5' site of tPA gene, the expression cassette was prepared and transformed into a strain with manipulated oxidizing cytoplasm. To express the protein of interest, induction was made and the successful expression of functional Tpa confirmed by SDS-PAGE analysis and gelatin hydrolysis. So, complex proteins can be produced in *E. coli* using both cytoplasm and periplasm cumulative power (78).

Cytoplasm reducing environment seems to play a key role in inappropriate folding of such high disulfide bonded proteins. So, it is one of the factors affecting the efficiency of such complex proteins production (26-29). The oxidizing environment of periplasm of *E. coli* is more, and in wild-type bacteria is more satisfactory than cytoplasm for proper folding (2).

Due to genetic simplicity, fast growth rate, high cell density production and the availability of an increasingly large number of vectors and host strains (2-4), *E. coli* prokaryotic systems have been the most widely used systems for the recombinant protein production. Manipulating the condition of cytoplasm by converting its reducing nature into an oxidizing environment and the secretion of the protein into the less reducing environment of periplasm are two common *in vivo* approaches to improve the conformation of complex proteins in *E. coli*. The recombinant protein maintained in an oxidizing environment of cytoplasm and the proper conformation of the protein forms when the gene of interest, without the signal peptide encoding sequence, is transformed into the engineered *E. coli* strain (30).

In another approach, the gene of interest containing a suitable signal peptide is introduced to the bacterial host, and the signal peptide directs the protein into the periplasm. Thus, the secretion of the protein into the less reducing environment of periplasm takes place. The potential of using a signal peptide for production of a highly disulfide-bonded protein in an *E. coli* strain with engineered cytoplasm has been carried out and recommended for use in commercial production of the protein.

Expression of Recombinant tPA

Addition of IPTG induces the expression of recombinant tPA. A selected positive transformant was grown in LB medium, until the cell optical density (OD, 600 *nm*) reached 0.3-0.5 (78).

Following induction, four hour- samples were taken and the lysates of induced and non-induced cells were compared in a standard SDS-Polyacrylamide gel and checked by gelatin hydrolysis test. Both plasminogen and gelatin co-polymerized and immobilized with a non-reducing polyacrylamide gel in the zymography test. Before and after induction, cell lysate of transformed host was applied to conform and evaluate activity of recombinant tPA. The transparent regions on the gel, which was observed only in a transformed host, indicated that the plasminogen is digested by the serine-protease activity of t-PA and the derived plasmin resulted in gelatin hydrolysis. Actylase or commercial t-PA was used as positive control and the negative control was Cell lysate of transformed expression host, before induction.

Thus, in this way, the protein of interest was produced in an oxidizing environment of cytoplasm and the disulfide bonds would be formed to some extent. Then, the produced protein transfers by the signal peptide into the periplasm where, a greater amount of the disulfide bonds would be formed in the periplasm with less reducing environment, and result in an active protein production.

CONCLUSIONS

Thrombolytic drugs play a crucial role in the management of patients with acute myocardial infarction, pulmonary embolism, deep vein thrombosis and arterial- thrombosis, acute thrombosis of retinal vessel, extensive coronary emboli, and peripheral vascular thrombo-embolism. In this study, the viable production of the biopharmaceutical reteplase from transgenic K2S plants takes place. Reteplase is indicated for the treatment of thrombosis and for use in the management of acute myocardial infarction (AMI) in adults, to improve ventricular function following AMI, reduce the incidence of congestive heart failure, and reduce mortality associated with AMI.m The risk of transgene dispersion in transplastomic plants is low due to the maternal mode inheritance, the cell culture system guarantees transgene containment. Also, like other bioreactor systems, the target compounds can be produced under strictly controlled culture conditions, thus avoiding the risk of contamination with human pathogens associated with mammalian cells. It also has the capacity for proper protein folding. These results open a new avenue to produce reteplase in transplastomic plant cell cultures as an alternative bio-sustainable system for biopharmaceutical production. Considering the potential advantages of plant cell cultures to produce biopharmaceuticals and demonstrate for the first time, the capacity of cell suspension cultures derived from K2S transplastomic tobacco plants for the bioactive target peptide production. It seems to work quickly and has lower bleeding risk than alteplase, also faster clot resolution occurs than alteplase. This may be re-administered as necessary, as it is not antigenic and almost never associated with any allergic manifestation. On the other hand, *Pichia pastoris* KM71H proved to be an appropriate strain for production of active recombinant protein. The approximate size of 70 kDa for recombinant t-PA was revealed by Western blotting test. Another is expression of Human Tissue Plasminogen Activator in

Escherichia coli using Cytoplasmic and Periplasmic Cumulative Power. It is one of the desirable methods for this biopharmaceutical production. The protein of interest was produced in an oxidizing environment of cytoplasm and the disulfide bonds would be formed to some extent. So, these are the methods of better production, and have various potential applications in medical field. Thus, tPA play better role in the management of patients with acute myocardial infarction, pulmonary embolism.

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